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13. ABSTRACT (Maximum 200 words)

The primary objective of the research performed under this grant was to gain a fuller understanding of the requirements for export of heterologous proteins from Escherichia coli. The long term goal would be to engineer bacteria to secrete cloned proteins to the periplasmic space either to improve the overproduction and purification of such proteins or to facilitate bioremediation efforts using bacteria that can export degradative enzymes.

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Secretion of Heterologous Proteins from *Escherichia coli*

Final Progress Report

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U.S. Army Research Office

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Statement of the Problem Studied

The primary objective of the research performed under this grant was to gain a fuller understanding of the requirements for export of heterologous proteins from *Escherichia coli.* The long term goal would be to engineer bacteria to secrete cloned proteins to the periplasmic space either to improve the overproduction and purification of such proteins or to facilitate bioremediation efforts using bacteria that can export degradative enzymes.

Export of proteins to the periplasm or outer membrane is carried out by the Sec pathway [1,2]. Such exported proteins, called secretory proteins, are synthesized with an amino terminal tail, the signal sequence, that directs them to the export pathway. Mutations to the signal sequence drastically reduce export of secretory proteins. However, suppressor mutations have been identified in the Sec proteins that restore export of such signal sequence defective secretory proteins [3-5]. Indeed, even a secretory protein with a complete deletion of the signal sequence is exported to a reasonable level (~30%) in a *prl* suppressor strain [6,7]. Previous analyses of the *prl* suppressors led to a mechanistic model in which the wild type SecE and SecY proteins are proposed to function as proofreaders, rejecting secretory proteins with defective signal sequences from the export pathway. The Prl suppressor forms of SecE and SecY are thought to be defective in this proofreading function, thereby allowing export of secretory proteins with mutations in, or even deletions of, the signal sequence [7,8].

Even though Prl suppressors promote the export of secretory proteins that completely lack a signal sequence, no mislocalization of cytoplasmic proteins has been observed in *prl* strains. That is, proteins that are supposed to remain cytoplasmic are not mislocalized to the periplasm [9]. The central question addressed in this research is how the bacterial cell is able to differentiate between cytoplasmic proteins and secretory proteins with no signal sequence. It has been suggested that perhaps secretory proteins fold more slowly in the cytoplasm than do cytoplasmic proteins, allowing time for SecB, the export-specific chaperone, to bind and target the secretory to the export machinery [10]. In a *prl* suppressor strain, any protein thus targeted to translocase would be exported. Therefore, SecB binding and targeting would be the limiting factor for export of non-secretory proteins in a *prl* suppressor strain. We proposed that it should be

possible to engineer proteins to become a substrate for SecB and allow export of those proteins in a *prl* strain. To determine the requirements for SecB binding, the following specific goals were addressed:

(i) Does a slow-folding mutant of a cytoplasmic protein become a substrate for SecB binding, and therefore, for export?

(ii) What constitutes a SecB binding site? And will addition of a SecB binding site enhance export of a non-secretory protein?

Summary of most important results

The first goal of this research was to investigate the role of protein folding in export. We approached this problem by analyzing cytoplasmic protein containing a mutation that results in a slow folding protein. The protein selected was a well characterized fragment of λ repressor [11]. The wild type form of this fragment folds rapidly into a stable three dimensional structure, while the mutant that we used remains approximately 60% unfolded under the physiological conditions employed. We predicted that the unfolded nature of the protein would result in SecB binding and targeting to translocase. In a *prl* strain, we expected to observe export of a fraction of the repressor protein. This was not the result we observed; we did not detect any export of the λ repressor, even in a *prl* suppressor strain.

There are two possibilities for this observation: 1) the unfolded protein is not recognized and bound by SecB or 2) SecB binding is not sufficient to result in targeting to translocase. It is important to our understanding of protein export to distinguish between these alternative possibilities. Therefore we assessed binding of the slow folding λ repressor to SecB by two different methods. We used co-immunoprecipitation to detect in vivo complexes and protease protection assays to observe binding in vitro. In both cases, no complex formation was observed between λ repressor and SecB. These results demonstrate that slow folding is not sufficient for SecB binding. This is an important result as some of the current literature suggests that SecB is able to bind any unfolded protein [12-14]. We have concluded from these experiments that there is additional information within a secretory protein that targets it to translocase. To engineer heterologous proteins for export, it is crucial that we be able to identify these additional determinants.

In order to expand the number of possible SecB substrates we could examine, we have initiated a genetic screen to identify mis-folded proteins that are exported in a *prl* suppressor strain. The screen utilizes a strain we constructed which is deficient in three cytoplasmic peptidyl prolyl isomerases (PPIs). These PPIs assist in protein folding; therefore the mutants should contain a number of substrate proteins that are unfolded, mis-folded, or slow to fold. We then introduce random *phoA* fusions to detect proteins that are exported when *prlA4* is expressed, and are not exported when *prlA4* is not

expressed. We have begun our screening and identified several fusions that answer the initial criterion. So far, however, all have failed subsequent control tests (we have designed screens to eliminate fusions to genes that are either subject to catabolite repression or are induced by arabinose - both situations that would answer the initial screen). Although the first fusions did not pass all screens, it is encouraging that we obtained fusions of the sorts we predicted. These experiments are continuing with alternative funding sources.

The second major goal of this project was to identify SecB binding sites through the use of phage display. This technique is based on the ability of filamentous bacteriophage, such as M13, to tolerate genetic fusions to coat proteins. The hybrid fusion is inserted into the phage coat, resulting in the "display" of the fusion on the phage surface where it is accessible for binding by other proteins. By constructing a random library of amino acids fused to the coat protein, one can effectively screen for those sequences which bind to the protein of interest, in this case, SecB.

We have identified 12 individual dodecamers (from 28 separate phage) that bind SecB when presented on phage. Analysis of these sequences has not revealed any obvious common characteristics. The most promising so far seems to be an abundance of aromatic residues or structure disrupting amino acids (Trp, Tyr, His, Pro). We also utilized a library that expressed 38 amino acid peptides, but found results to be even less enlightening. We are continuing to select and sequence phage from the 12-mer library with the intent of identifying statistically significant over-representation of particular amino acids. We have also synthesized two of the peptides and will use these free peptides in binding assays to verify that they do in fact bind to SecB.

In summary, the results that we obtained indicate that 1) slow folding is not the sole determinant for SecB binding, and 2) the amino acid sequence to which SecB binds is not a simple, conserved sequence, but is composed primarily of aromatic residues. It is clear that secretory proteins contain information in addition to the signal sequence and slow folding characteristics that target them to the secretory apparatus. Future studies will be focussed on identification of these sequences.

List of all publications and technical reports

Mallik, I., Smith, M.A. & Flower, A.M. "Slow Folding of a Cytoplasmic Protein is Not Sufficient for SecB Binding" Submitted to FEBS Letters, December 15, 2000.

List of all participating scientific personnel showing any advanced degrees earned by them while employed on this project

Ipsita Mallik, earned M.S., 1997

Kathleen LaVoi

Margaret Smith

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Report of inventions

None

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